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van der Meer, S.F.T.; Jaspers, R.T.; Jones, D.A.; Degens, H.

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TIME-COURSE OF CHANGES IN THE MYONUCLEAR DOMAIN DURING DENERVATION IN YOUNG-ADULT AND OLD RAT GASTROCNEMIUS MUSCLE

SANDER F.T. VAN DER MEER, MSc,[1,2*] RICHARD T. JASPERS, PhD,[3] DAVID A. JONES, PhD,[4] and HANS DEGENS, PhD[1,5]

[1] Institute for Biomedical Research into Human Movement and Health, Manchester Metropolitan University, John Dalton Building, Oxford Road, Manchester M1 5GD, UK

[2] Institute of Aerospace Medicine, Division Space Physiology, German Aerospace Center, Cologne, Germany

[3] Research Institute MOVE, Faculty of Human Movement Sciences, VU University Amsterdam, Amsterdam, The Netherlands

[4] School of Sport and Exercise Sciences, University of Birmingham, Birmingham, UK

[5] Department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

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ABSTRACT: If myonuclear loss initiates muscle wasting, it should precede the loss of muscle mass. As aging affects muscle plasticity, the time-course of muscle atrophy during disuse may differ between young and old animals. To investigate this, gastrocnemius muscles of 5- and 25-month-old rats were exposed to 1, 2, or 4 weeks of denervation, whereas the contralateral gastrocnemius muscles served as controls. Muscle fibers of each type responded similarly to 4 weeks of denervation. For both ages most of the atrophy (36%; $P < 0.001$) occurred in the first 2 weeks. In young-adult muscles, the myonuclear number remained constant, but in old muscles it decreased to below control level after 4 weeks of denervation ($P < 0.05$). Despite this differential response, myonuclear domain size decreased similarly at both ages ($P < 0.001$). In both young-adult and old rats, denervation-induced atrophy was not preceded by a loss of myonuclei.

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According to the concept of the myonuclear domain, muscle fiber hypertrophy is associated with a gain, and atrophy is associated with a loss of myonuclei; therefore, a constant volume of cytoplasm per myonucleus is maintained.[1] This notion has been supported by many,[2–6] but not all,[7,8] studies that report disuse-induced atrophy is accompanied by a concomitant reduction in the number of myonuclei per fiber. It should be noted, however, that disuse atrophy occurs rather rapidly. For instance, about half of the atrophy of rat skeletal muscle was found to occur during the first weeks after denervation.[9,10] Thus, although the number of myonuclei may already be reduced after 10–14 days of denervation or unloading,[2–4] it is not clear whether this precedes or lags behind muscle fiber atrophy.

Myonuclear domain size depends on fiber type. Type I and IIA fibers have a smaller myonuclear domain size than IIB fibers,[11] and it is inversely related to the oxidative capacity of the fiber.[12] This difference may be related to a generally faster protein turnover and a higher demand for mRNA transcription of mitochondrial proteins in high oxidative muscle fibers. Recently, it has been shown that the relative loss in oxidative capacity of denervated muscle transiently exceeds the loss of muscle mass.[9,10] An equivalent loss of myonuclei in low oxidative (glycolytic) type IIB and (high oxidative) type I and IIA fibers implicates a higher relative impact on the rate of transcription of contractile proteins in these latter fiber types, which may explain the more rapid denervation-induced atrophy in fast than in slow muscles.[13]

The structural and functional changes observed in the myonuclei of old rat muscle suggest that the transcriptional efficiency of the myonucleus is reduced at an older age.[14] A reduced transcriptional efficiency may at least partly explain the age-related muscle atrophy. The increased number of myonuclei per fiber at older ages[15–17] may compensate for the reduced transcriptional efficiency per myonucleus and be reflected by a reduction in the myonuclear domain size.

In addition to a reduced transcriptional efficiency of the myonucleus there is also evidence for elevated nuclear apoptosis[18,19] and a decline in the number of satellite cells.[16,20–23] Denervation is associated with an increase in apoptosis[24,25] that is even more marked in old muscles.[26] As a consequence, old muscle fibers are probably more vulnerable to loss of myonuclei than young-adult muscle fibers, and they may be less able to maintain the myonuclear domain size. These circumstances may make old muscles more vulnerable than young muscles to disuse-induced atrophy, such as that induced by denervation, unless satellite cell proliferation is more enhanced in old than in young muscle.

The objective of this study was to determine the time-course of changes in the myonuclear domain size of different fiber types during denervation-induced atrophy in young and old rat gastrocnemius muscle. Muscle denervation was chosen because it causes a more rapid and pronounced atrophy than other disuse models, such as tenotomy and spinal cord injury.[27,28] We hypothesized that

Abbreviations ANOVA, analysis of variance; ATPase, adenosine-5′-triphosphatase; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; FCSA, fiber cross-sectional area; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline plus Tween; WGA, wheat-germ agglutinin

*Deceased

Key words: aging, atrophy, myonuclei, RNA, skeletal muscle

Correspondence to: S. F. van der Meer; e-mail: sander.vandermeer@dlr.de

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denervation in old muscles is accompanied by a more rapid and larger loss of myonuclei than in young muscles and, consequently, by a faster rate of atrophy.

Because denervation-induced atrophy has been reported to be more pronounced in fast than slow muscles,[13] we determined the myonuclear domain size of three different fiber types in the rat gastrocnemius muscle during 4 weeks of denervation. We hypothesized that type IIB/X fibers atrophy more than type I fibers and that the size of the myonuclear domain decreases more in type IIB/X than in type I fibers.

METHODS

Animals. Male Wistar rats, 5 or 25 months of age at the time of the terminal experiment, were housed in pairs with free access to food and water. The environment was maintained at 22°C on a 12-h light/12-h dark cycle. The 5-month-old (young-adult) rats have ended their main growth spurt and are mature, whereas the 25-month-old rats are considered old; the Wistar rat has a median lifespan of 21.5 months (Harlan Laboratories, Indianapolis, Indiana) and a mean life expectancy of around 24 months.[29,30] Rats were randomly assigned to groups in which the left gastrocnemius muscle was subjected to 1, 2, or 4 weeks of denervation, whereas the right gastrocnemius muscle served as an internal control, as described previously.[9] Briefly, rats were anesthetized with isoflurane and, under aseptic conditions, the branches of the n. ischiadicus to the gastrocnemius and soleus muscles were cut and sewn into the biceps femoris to prevent reinnervation. Rats received a subcutaneous injection of carprofen (0.5 mg/kg) after surgery as an analgesic. To prevent bias related to age differences within groups, the denervation was timed so that all animals in the young-adult group were 5 months old and those of the old group were 25 months old when the animals were killed, irrespective of whether their muscles were subjected to 1, 2, or 4 weeks of denervation. The animals were killed by an intraperitoneal injection of an overdose of pentobarbital sodium, and the gastrocnemius muscles were quickly excised, blotted dry, and weighed on an analytical balance. Muscles were then slightly stretched, pinned on cork, frozen in liquid nitrogen, and stored at −80°C until analysis. All procedures were approved by the local research ethics committee of the Radboud University Nijmegen Medical Centre.

Immunochemistry. Cross-sections (10 μm) were cut from the midbelly of the gastrocnemius muscle on a cryostat at −20°C, air-dried, and stored at −80°C. To distinguish type I, IIA, and IIB/X muscle fibers, cross-sections were stained for myosin adenosine-5′-triphosphatase (ATPase) after pre-incubation at pH 4.55.[31] From a mixed region of the cross-section, approximately 50 fibers of each fiber type were selected for analysis (Fig. 1A). Serial sections were stained for pax7 (Fig. 1B), a (paired box) transcription factor that is expressed in quiescent, activating, and proliferating satellite cells.[32] To do this, sections were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes, washed with PBS plus Tween (PBST), and blocked in 10% normal swine serum in PBS for 30 minutes before incubation for 60 minutes with mouse monoclonal pax7 antibody [3.6 μg/ml in 0.1% bovine serum albumin (BSA)–PBS, developed by A. Kawakami and obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the Department of Biology, University of Iowa, Iowa City, Iowa]. As a negative control, one slide was treated in the same way, except for omission of incubation with the primary antibody. After washing in PBST, sections were incubated in the dark for 30 minutes with Alexa Fluor 488 goat anti-mouse secondary antibody (20 μg/ml in 0.1% BSA–PBS; Invitrogen, Breda, The Netherlands) and washed with PBST. To stain the basal lamina, the sections were subsequently incubated in the dark for 20 minutes with Texas red–wheat germ agglutinin (WGA) conjugate (20 μg/ml in PBS WGA; Invitrogen). After final washes with PBS the sections were embedded using mounting medium (Vectashield HardSet) with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, California) to visualize all nuclei in the cross-section. Images were captured at 10× magnification using a CCD camera (PCO; Sensicam, Kelheim, Germany) connected to a fluorescence microscope (Axiovert 200M; Zeiss, Göttingen, Germany) with image processing software (Slidebook 4.1; Intelligent Image Innovations, Denver, Colorado).

Data Analysis. The immunofluorescent images were used to count the myonuclei and satellite cells and to measure the fiber cross-sectional area (FCSA) of the type I, IIA, and type IIB/X fibers. Nuclei were considered to be myonuclei if they were located within the basal lamina unless they were pax7-positive, in which case they were classified as satellite cell nuclei (Fig. 1C). Myonuclei not immediately beneath the sarcolemma were regarded as central myonuclei. All counting was carried out by the same researcher, who was blinded to the age or condition of the muscle. Image size was calibrated using a slide micrometer, taking the pixel aspect ratio into account. The public domain software package ImageJ (W.S.

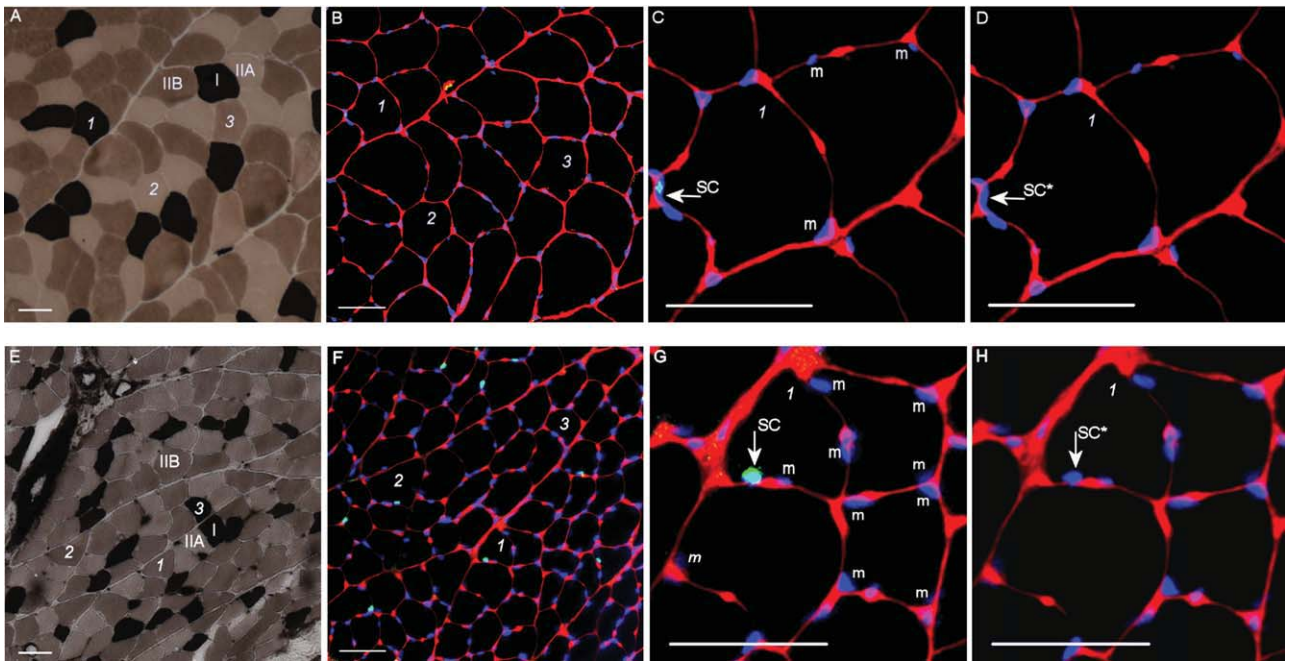

FIGURE 1. Typical stained gastrocnemius muscle sections of control **(A–D)** and 4-week-denervated **(E–H)** muscles from young-adult rats. **(A, E)** Sections stained for myosin ATPase after pre-incubation at pH 4.55 with black-stained fibers ('I') identified as type I fibers; white ('IIA') indicates type IIA fibers, and brown staining ('IIB/X') indicates type IIB/X fibers. Numbers ('1–3') indicate same muscle fiber in serial section (B) [serial section of **(A)**] and **(F)** [serial section of **(E)**] stained for the basal lamina [wheat germ agglutinin (WGA) in red], nuclei [4′,6-diamidino-2-phenylindole (DAPI) in blue], and satellite cells (pax7 in green). **(C, G)** Magnified selection of image **(B)** and **(F)**, respectively, in which the number '1' indicates the same muscle fiber shown in **(B)** and **(F)**; nuclei (blue) were identified as myonuclei if they were inside the red-stained basal lamina ('m'), unless they were pax7-positive, in which case they were identified as satellite cells (turquoise-green, 'Arrow + SC'). Images **(D)** and **(H)** represent **(C)** and **(G)**, respectively, but without pax7 staining, and therefore show that the identification of satellite cells was conducted with strict colocalization of pax7 and DAPI ('Arrow + SC*') inside the ring of the basal lamina. Bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Rasband, ImageJ, U.S. National Institutes of Health, Bethesda, Maryland; http://rsb.info.nih.gov/ij/, 1997–2007) was used to trace the WGA-stained basal lamina of the muscle fibers to compute the FCSA. To express the number of myonuclei per millimeter fiber length we used a modification of a previously described method,[33,34] assuming a nuclear length of 17 μm, as observed in single muscle fibers of the gastrocnemius muscle (data not shown). Given a nuclear length of 17 μm, a section thickness of 10 μm, and the fact that the smallest detectable nuclear fragment is 1 μm, the number of nuclei per unit fiber length was divided by 2.75 to correct for multiple counting. The actual conversion into myonuclear number for a given length of fiber ($m_{f,l}$) was calculated as:

$$m_{f,l} = \frac{m_{f,c} \times L_f}{D \times 2.75}$$

where $m_{f,c}$ is the number of myonuclei in a muscle fiber cross-section, L_f is the length of the muscle fiber segment in microns (in this case 1000, to present the results as myonuclei per millimeter of fiber length), and D is the thickness of the cross-section in microns. Myonuclear domain size was calculated by dividing the mean FCSA by the mean number of myonuclei per millimeter of muscle fiber length.

RNA and Protein Concentration. The RNA pool in skeletal muscle fibers consists of approximately 80% ribosomal RNA and thus gives a rough estimate of the translational capacity.[35] Changes in the total RNA content (in micrograms of RNA in the muscle as a whole) during the time-course of denervation would reflect changes in the translational capacity and total transcriptional activity in the muscle. Changes in the concentration of RNA (in micrograms of RNA per milligram muscle) show whether the rate of transcription per unit muscle mass is altered, whereas the RNA-to-myonucleus ratio (micrograms of RNA per myonucleus) gives an indication of the transcriptional activity of individual myonuclei. The ratio of the protein and RNA concentration (subsequently referred as the protein-to-RNA ratio) has been determined to provide insight into the rate of translation.[36]

To isolate RNA, 25 30-μm cross-sections of the whole gastrocnemius muscle were cut and collected in pre-cooled microcentrifuge tubes. The samples were then pre-weighed (mean ± SEM: 55.1 ± 4.6 mg) while frozen. The weight of each

Table 1. Effect of denervation and age on gastrocnemius muscle mass and gastrocnemius muscle mass normalized to body mass.

	Body mass (g)		Muscle mass (mg)		GM to BM (mg/g)	
Duration (*n*, Y / *n*, O)	Y	O	Y	O	Y	O
0 week (15 / 10)	442 ± 7	608 ± 21	2231 ± 130	2218 ± 214	5.1 ± 0.3	3.7 ± 0.2†
1 week (5 / 3)	449 ± 10	584 ± 13	1724 ± 57*	1642 ± 142**	3.9 ± 0.2*	2.8 ± 0.3**††
2 weeks (5 / 3)	439 ± 10	610 ± 45	1269 ± 39*	1313 ± 96*	2.9 ± 0.1*	2.2 ± 0.1*†
4 weeks (5 / 4)	439 ± 14	630 ± 53	732 ± 107*	1174 ± 315*	1.7 ± 0.3*	1.9 ± 0.5*
ANOVA effects						
Interaction	NS		$P = 0.01$		$P < 0.001$	
Age	$P < 0.001$		NS		$P < 0.001^{\dagger}$ and $P < 0.01^{\dagger\dagger}$	
Duration	NS		$P < 0.001^{*}$ and $P < 0.01^{**}$		$P < 0.001^{*}$ and $P < 0.01^{**}$	

Y, young adult; O, old; n, number of observations; YGM to BM, gastrocnemius muscle mass/body mass ratio; body mass values at 0 week are the average taken of all young-adult and old animals' body masses at the end of the denervation experiment and can therefore not be regarded as a control level. NS, not significant; P-values indicate main effects, unless an interaction effect was found, in which case P-values indicate the results of post hoc tests.
Different from control at: $^{*}P < 0.001$ and $^{**}P < 0.01$.
Different from young-adult at: $^{\dagger}P < 0.001$ and $^{\dagger\dagger}P < 0.01$.

tube was determined before the sample was added. The samples were then homogenized on ice in 1 ml of TriReagent (Molecular Research Center, Cincinnati, Ohio) using a basic homogenizer (Ultra Turrax T18; IKA, Staufen, Germany). The RNA concentration (micrograms per milligram of gastrocnemius muscle) was determined in triplicate with a photospectrometer (Biowave II; Biochrom, Ltd., Cambridge, UK) and multiplied with the gastrocnemius muscle mass to obtain the total RNA content (micrograms).

For determination of the protein concentrations separate samples of each gastrocnemius muscle were collected (mean ± SEM: 39.3 ± 1.5 mg) as described earlier for the isolation of RNA. The samples were homogenized on ice in 400 μl of radio-immunoprecipation assay (RIPA) buffer containing protease inhibitors. Subsequently, the protein concentration (in milligrams per milligram gastrocnemius muscle) was measured in triplicate by using bicinchoninic acid reagents (Pierce, Rockford, Illinois). The protein-to-RNA ratio was calculated as the muscle protein concentration divided by the muscle RNA concentration.

Statistics. The effects of age and duration of denervation on gastrocnemius muscle mass and RNA and protein concentrations were assessed using a two-way analysis of variance (ANOVA). The factor "age" had two levels (young-adult or old), and the factor "duration" had four levels [0- (contralateral control muscle), 1-, 2-, and 4-week denervation]. A three-way ANOVA was performed to test for effects of age, duration of denervation, and fiber type on cellular changes in gastrocnemius muscle, with the factor "fiber type" subdivided into three levels (type I, type IIA, and type IIB/X). Significant fiber-type effects were further analyzed by a Bonferroni-corrected post hoc test to assess which fiber types differed from each other. Whenever a significant duration effect was found, a one-sided Dunnett's test was performed to determine which time-points differed from the control situation. If significant interactions between two or more factors were found, one-way ANOVAs were subsequently conducted to investigate the location of the difference. In case a parameter did not have equal variances in the different conditions, the data were first transformed by taking the $\log^{10}$ of the data. Data are presented as mean ± SEM unless stated otherwise. Effects were considered significant at $P < 0.05$.

RESULTS

Age and unilateral denervation may have an effect on the contralateral leg and influence the interpretation of the data.[37] To assess the possible effect of age and denervation on the contralateral gastrocnemius muscle we performed a two-way ANOVA on all control (nerve-intact) muscles with age and duration of denervation (in this case three levels, i.e., at 1, 2, or 4 weeks of denervation) as grouping factors. There were no significant effects of age, duration of denervation, or interactions between these two factors in any of the measured parameters. This indicates that denervation did not significantly affect the contralateral gastrocnemius muscle. Therefore, we pooled the data of the contralateral (nerve-intact) gastrocnemius muscle for the young-adult and old rats and presented them as the 0-week time-point.

Body Mass and Muscle Mass. The body mass of old rats was 37% higher than that of young-adult rats ($P < 0.001$; Table 1). Gastrocnemius muscle mass did not differ between old and young-adult rats, and denervation resulted in significant atrophy, which occurred mainly after 1 (25%) and 2 weeks (42%) of denervation ($P < 0.001$; Table 1). The interaction between age and duration of denervation ($P = 0.01$; Table 1), however, indicates that

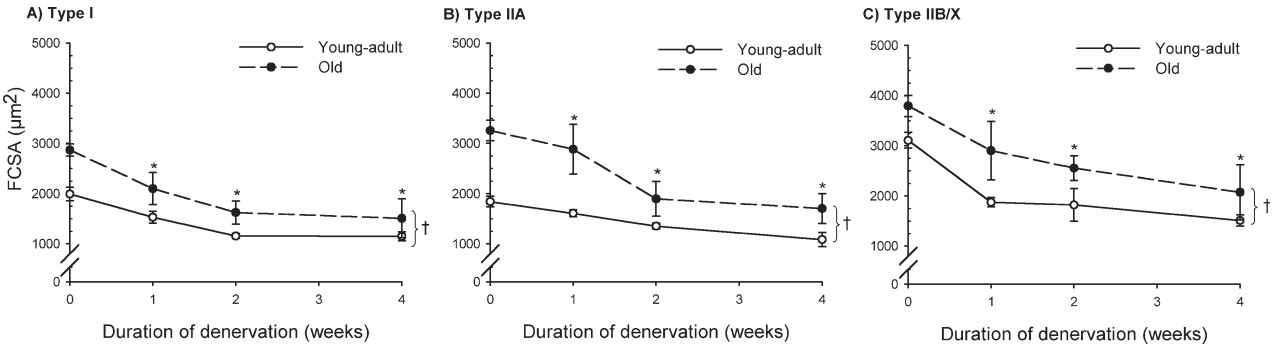

FIGURE 2. Effect of 4 weeks of denervation, age, and fiber type on fiber cross-sectional area (FCSA) of gastrocnemius muscles from young-adult and old rats. **(A)** Type I, **(B)** type IIA, and **(C)** type IIB/X muscle fibers. Effect of duration of denervation ($P < 0.001$): *at specific time-points, denervated muscle fibers were smaller than control (nerve-intact) muscle fibers ($P < 0.001$). Effect of age ($P < 0.001$): †muscle fibers from old animals were larger than those of young-adult animals. Effect of fiber type: type IIB/X fibers **(C)** were larger than type I **(A)** and IIA **(B)** fibers ($P < 0.001$).

the rate of atrophy differed between young-adult and old animals. This interaction is reflected by a similar initial muscle mass in young-adult and old animals, and by 67% vs. 47% atrophy after 4 weeks of denervation in young-adult and old animals, respectively.

Due to the larger body mass of the old animals, the gastrocnemius muscle mass/body mass (GM-to-BM) ratio was lower in old compared with young-adult animals at all time-points, except at 4 weeks of denervation ($P < 0.001$; Table 1). The effect of denervation followed the same time-course as described earlier for muscle mass.

Fiber Cross-Sectional Area. Three-way ANOVA showed that, irrespective of age and duration of denervation, the FCSA of type IIB/X fibers was significantly larger than that of type I and type IIA fibers ($P < 0.001$), whereas the FCSA of type I and IIA fibers did not differ (Fig. 2).

In the control gastrocnemius muscles the FCSA was 37% larger in old than in young-adult muscles ($P < 0.001$; compare Fig. 2A and B). Denervation caused a progressive decrease in FCSA ($P < 0.001$; Fig. 2), with an approximately 25% reduction in FCSA at the first week, 36% at the second week, and 47% at the fourth week after denervation. This time-course of atrophy was similar in young-adult and old rats and did not differ between fiber types.

Myonuclear Number. The interaction between age and duration of denervation ($P = 0.01$) indicates that the time-course of the change in myonuclear number differed between young-adult and old muscles. Old control muscle fibers contained 36% more myonuclei than young-adult control fibers, which also persisted at 1 and 2 weeks after denervation ($P < 0.001$). This difference had disappeared, however, after 4 weeks of denervation. The number of myonuclei in old gastrocnemius was significantly lower by 4 weeks of denervation compared with control ($P < 0.05$), whereas there was no change in young myonuclear number (Fig. 3). We also observed a main effect of fiber type ($P < 0.001$); type IIA fibers contained 17% fewer

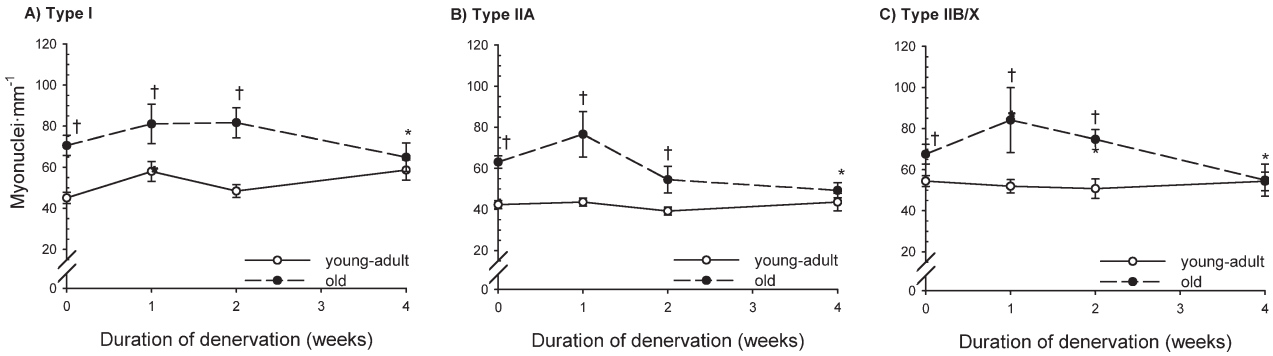

FIGURE 3. Effect of 4 weeks of denervation, age, and fiber type on number of myonuclei per millimeter fiber length of gastrocnemius muscles from young-adult and old rats. **(A)** Type I, **(B)** type IIA, and **(C)** type IIB/X muscle fibers. The duration of denervation × age interaction ($P = 0.01$) was reflected by an effect of duration of denervation in old ($P < 0.01$), but not in young-adult muscles. *At specific time-points, denervated muscle fibers contained fewer myonuclei per millimeter fiber length than control (nerve-intact) muscle fibers ($P < 0.05$). †Significantly different from corresponding young-adult muscle ($P < 0.001$). Effect of fiber type ($P < 0.001$): type IIA fibers **(B)** contained fewer myonuclei per millimeter fiber length than type I **(A)** and IIB/x (**C**) fibers ($P < 0.001$).

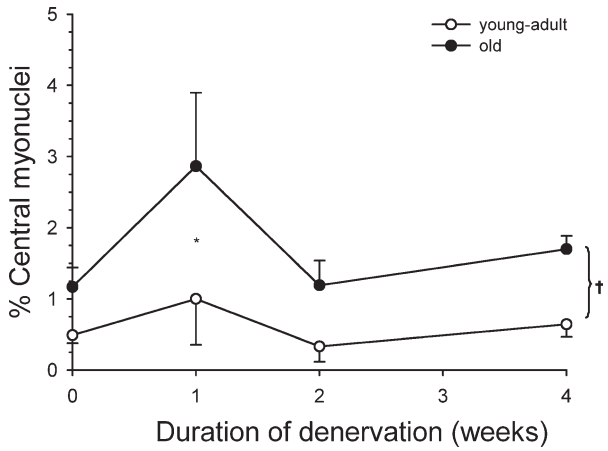

FIGURE 4. Effect of 4 weeks of denervation and age on percent central myonuclei [i.e., (total of central myonuclei per muscle / total of myonuclei per muscle) * 100]. †Effect of age ($P < 0.001$); effect of duration of denervation ($P < 0.05$): *at specific time-points, denervated muscles had a significantly higher percentage of central myonuclei than control muscles ($P < 0.05$).

myonuclei than type I and IIB/X fibers ($P < 0.001$; Fig. 3).

Old muscle fibers contained, irrespective of duration of denervation, an average of 2.5-fold more central myonuclei than young-adult fibers ($P < 0.001$; Fig. 4). Denervation had a significant effect on the number of central myonuclei. In Figure 4 this can be seen as a transient increase in the number of central myonuclei at 1 week of denervation ($P < 0.05$).

Myonuclear Domain Size. Myonuclear domain size did not differ between young-adult and old muscles. The progressive decrease in myonuclear domain size in response to denervation followed a similar time-course as the decrease in FCSA for both young-adult and old fibers ($P < 0.001$; Fig. 5), and the main reduction (37%) was observed after 1 week of denervation ($P < 0.001$; Fig. 5). After 1 week of denervation, the myonuclear domain size was further decreased, but the magnitude of the additional decrease in domain size was smaller (Fig. 5). Fiber type significantly affected myonuclear domain size ($P < 0.001$); myonuclear domain size was smallest in type I and largest in type IIB/X fibers ($P < 0.05$; Fig. 5).

Satellite Cells. The interaction between age and duration of denervation for the number of satellite cells per fiber ($P < 0.05$; Fig. 6A) indicates a different response in young-adult and old muscles. This interaction is reflected by an increase in the number of satellite cells per muscle fiber in young-adult muscles after 4 weeks of denervation ($P < 0.01$), whereas in old muscles a transient increase in number of satellite cells per fiber was observed after 1 week of denervation ($P < 0.05$; Fig. 6A). The number of satellite cells per muscle fiber did not differ between young-adult and old control gastrocnemius muscle. Although this is a reflection of the total number of satellite cells, it is probably more relevant to express this in relation to the FCSA. The number of satellite cells per square millimeter also did not differ between young-adult and old control muscles (Fig. 6B), but it was significantly increased after 1 week of denervation ($P < 0.05$; Fig. 6B).

RNA and Protein Concentration. The concentration of total RNA (micrograms per milligram gastrocnemius muscle) did not differ between young-adult and old gastrocnemius muscles. There was a transient increase in RNA concentration at 1 week of denervation ($P < 0.01$; Fig. 7A), and RNA content of the muscle was maintained despite significant atrophy. Nevertheless, after 2 and 4 weeks of denervation the RNA content was decreased in both young-adult and old muscles ($P < 0.01$; Fig. 7B). The amount of total RNA per myonucleus was not affected by age (Fig. 7C). Denervation, however, resulted in a decrease in the amount of RNA (in

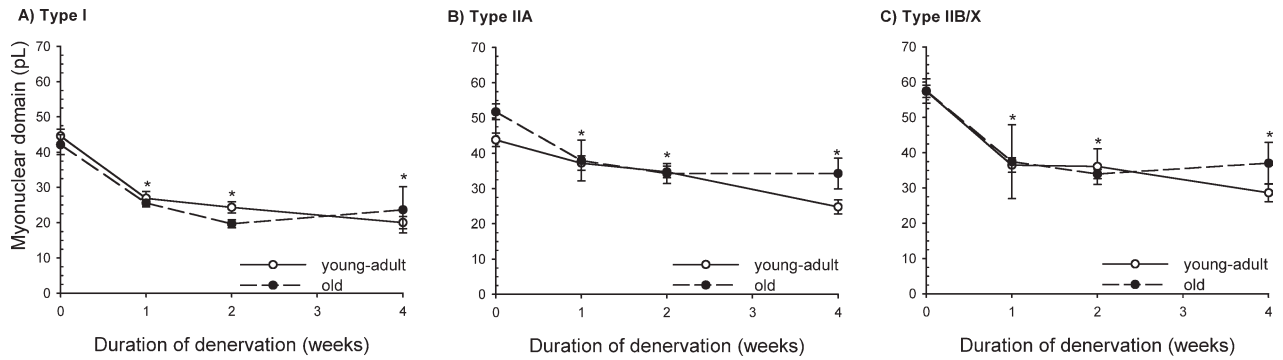

FIGURE 5. Effect of 4 weeks of denervation, fiber type, and age on myonuclear domain size of gastrocnemius muscles from young-adult and old rats. **(A)** Type I, **(B)** type IIA, and **(C)** type IIB/X muscle fibers. Effect of duration of denervation ($P < 0.001$): *at specific time-points, denervated muscle fibers had a smaller myonuclear domain than control (nerve-intact) muscle fibers ($P < 0.001$). Effect of fiber type ($P < 0.001$): myonuclear domain size was smaller in type I **(A)** than type II **(B, C)** muscle fibers ($P < 0.001$) and slightly larger in type IIB/X **(C)** than type IIA **(B)** fibers ($P < 0.05$).

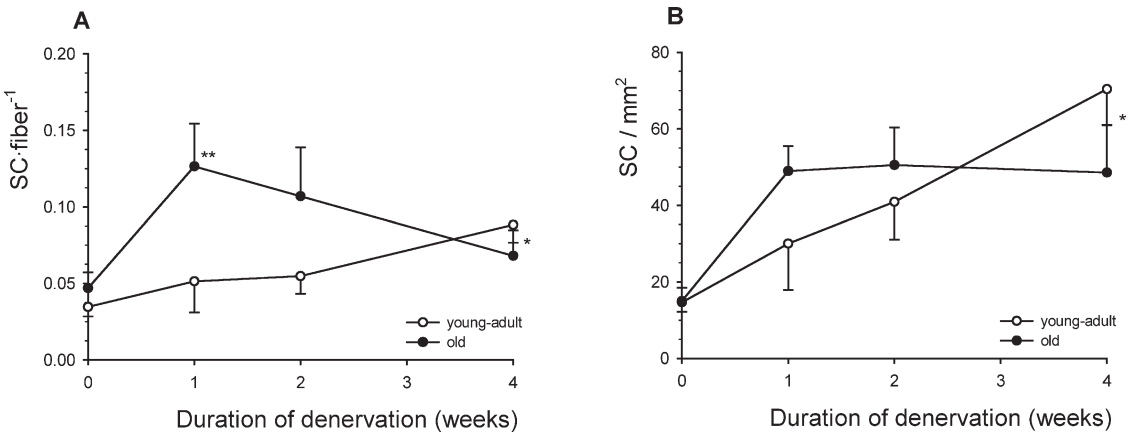

FIGURE 6. Effect of 4 weeks of denervation and age on number of satellite cells. **(A)** Number of satellite cells per muscle fiber. Age × duration of denervation interaction ($P < 0.05$): *different from control (nerve-intact) in young-adult muscles only ($P < 0.05$); and **different from control (nerve-intact) in old muscles only ($P < 0.01$). **(B)** Number of satellite cells per square millimeter of muscle fiber area. Duration effect ($P < 0.001$): *at specific time-points, denervated muscles contained more satellite cells per square of millimeter muscle fiber area than control (nerve-intact) muscles ($P < 0.05$).

micrograms) per myonucleus ($P < 0.01$), which became significantly different from control level after 2 and 4 weeks of denervation ($P < 0.05$; Fig. 7C).

Figure 7D and E shows that the protein concentrations of the gastrocnemius muscles and the protein-to-RNA ratios did not differ between young-adult and old animals, nor were they affected by 4 weeks of denervation.

DISCUSSION

The main findings of this study are that denervation-induced atrophy in young-adult rat gastrocnemius muscle was not accompanied by a reduction in the number of myonuclei per fiber. There was some loss of myonuclei in old muscles. However, in contrast to our hypothesis, the time-course of muscle fiber atrophy and decrease in myonuclear domain size was similar in young-adult and old rats and for both type I and type II fibers. These data do not fit the concept of the constant myonuclear domain. Interestingly, the number of satellite cells increased during denervation in both young-adult and old rats.

Time-Course of Denervation-Induced Atrophy. The time-course of denervation-induced atrophy of the gastrocnemius muscle fibers was similar in young-adult and old rats, where the bulk of the atrophy occurred during the first 2 weeks following denervation. It has been reported that type I fibers may even hypertrophy, at least in young-adult rats, which would underlie the differential rate of atrophy of denervated slow and fast muscles.[13,38] In our study, however, the degree and time-course of the denervation-induced atrophy was similar for type I and type II fibers. In addition, the time-course of muscle fiber atrophy of the gastrocnemius muscle was essentially the same as that of the soleus muscle[9] from the same animals as in our study. The discrepancy between our results and other studies for the rate of denervation-induced atrophy of type I fibers may be related to the type of muscles studied. In response to denervation of the flexor muscles, rats tend to drag their denervated limb forward with the ankle joint in extended position, with occasional mechanical flexing of the ankle. As a result, the extensor muscles may have been exposed to some stretch from the larger (denervated) flexor muscles[39] and thus be subjected to a small hypertrophic stimulus to, in particular, type I fibers. In fact, rat gastrocnemius muscles that are held at extended muscle length for 5 days while being denervated, showed a smaller reduction in type I myosin heavy chain mRNA than denervated muscles only.[40] This would explain why denervation resulted in atrophy in type I fibers of the soleus muscle, a plantar flexor like the gastrocnemius, whereas the type I fibers of the extensor muscles even hypertrophied.[13] Whatever the cause, even in the extensor muscles, the different rate of atrophy between fiber types had largely disappeared in the older muscles.[13]

In terms of muscle mass, the degree of atrophy after 4 weeks of denervation was greater in the young-adult than in old rats. This finding was in contrast to our hypothesis that old muscles would atrophy faster than young-adult muscles. However, as a consequence of the differential atrophy in young-adult and old muscles, the GM-to-BM ratio reached the same level in the old and young-adult muscles after 4 weeks of denervation. This corresponds with the suggestion made previously that, after cessation of neural input, the muscle reverts to a sort of "default" mass related to the body mass.[9]

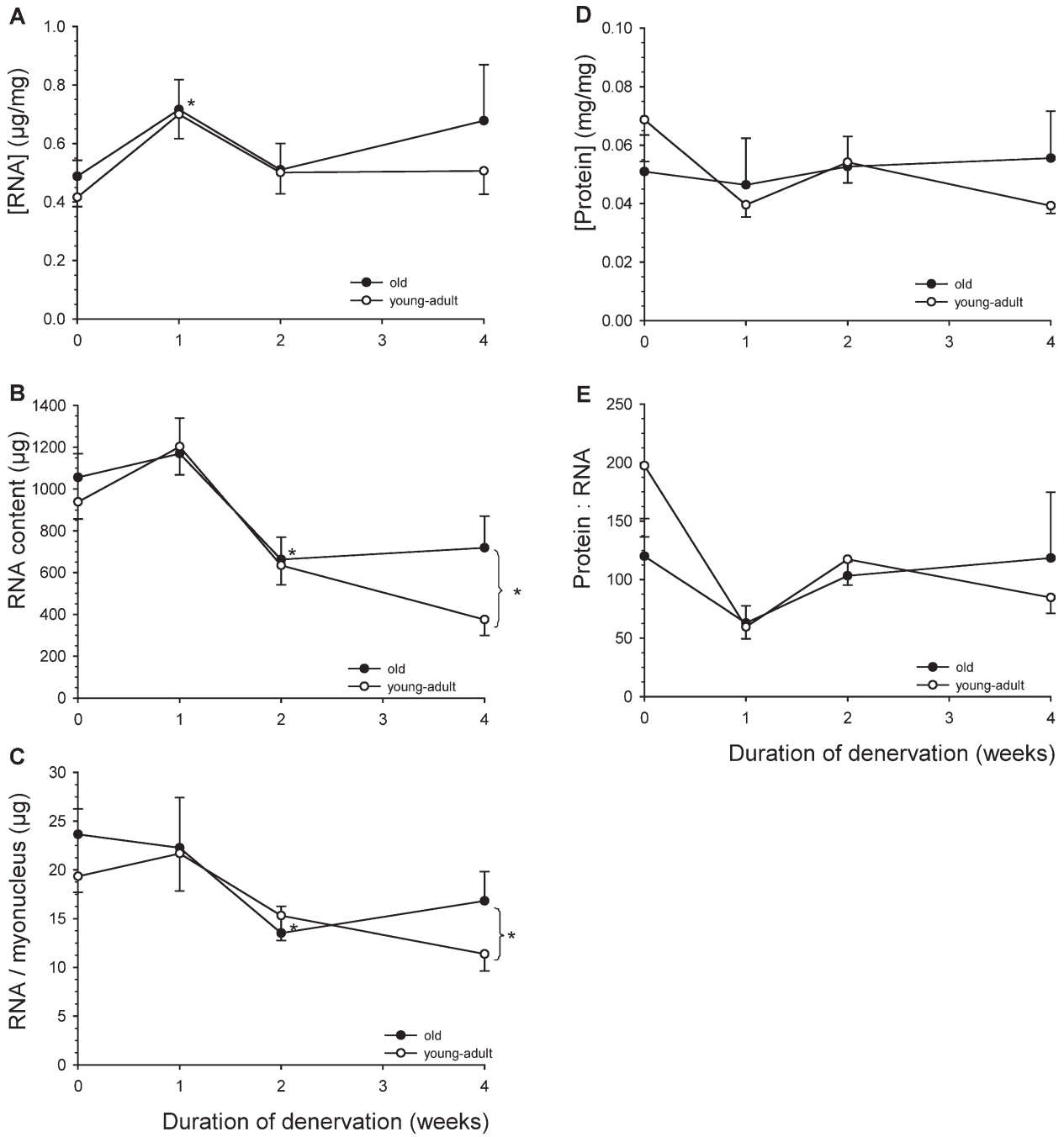

FIGURE 7. Effect of 4 weeks of denervation and age on RNA and protein concentrations. **(A)** RNA concentration (micrograms per milligram). Effect of duration of denervation ($P < 0.05$): *at specific time-points, [RNA] from denervated muscles was higher than control (nerve-intact) muscles ($P < 0.01$). **(B)** Total RNA content (micrograms) of gastrocnemius muscles. Effect of duration of denervation ($P < 0.001$): *at specific time-points, RNA content of denervated muscles was lower than that of control (nerve-intact) muscles ($P < 0.01$). **(C)** RNA content (micrograms) per myonucleus. Effect of duration of denervation ($P < 0.001$): *at specific time-points, RNA content per myonucleus of denervated muscles was lower than that of control (nerve-intact) muscles ($P < 0.01$). **(D)** Protein concentration (in milligrams per milligram). **(E)** Protein:RNA ratio.

Myonuclear Domain and Fiber Type. In agreement with previous findings,[12,41,42] we found that myonuclear domain size was lower in type I than in type IIA and IIB/X fibers. This was primarily due to the smaller number of myonuclei in type IIA and the larger FCSA of type IIB/X than type I fibers. There was only a small difference in myonuclear domain size between type IIA and type IIB/X fibers. The smaller myonuclear domain size in type I than in type II fibers is thought to be a reflection of the higher protein turnover rate in slow than in fast muscles,[43] due to their higher oxidative

capacity.[12] These differences in myonuclear domain size remained during the 4 weeks of denervation in both the young-adult and the old muscles, extending the observations in adult rat diaphragm denervated for 2 weeks.[42] This indicates that not only the decrease in FCSA but also the decrease in myonuclear domain size was independent of fiber type.

Effects of Aging on Myonuclear Number and Myonuclear Domain Size. The larger FCSA in old than in young-adult muscles in combination with the similar gastrocnemius muscle mass indicates that aging was accompanied by a loss of muscle fibers. This may have been due to a progressive loss of motoneurons during aging, which in turn may have caused a decrease in the number of muscle fibers (see Doherty[44] for review). This loss of fibers must be accompanied by loss of myonuclei and may underlie the often observed enhanced apoptosis in old muscles.[18,19] At the same time, however, old fibers had a 36% larger number of myonuclei per fiber, resulting in a similar myonuclear domain size in the fibers of young-adult and old gastrocnemius muscles. This suggests that aging is associated with myonuclear accretion. In this context, it is of interest to note that the expression of inhibitors of differentiation proteins, which play an important role in satellite cell proliferation and apoptosis, are elevated at old age.[18] Although one might therefore expect a larger number of satellite cells in old than in young-adult muscles this was not the case. It is likely that the process is too slow to result in a detectable increase in satellite cells. Indeed, although the number of central nuclei, indicative of regeneration, was larger in old than in young muscles, it was still less than 1.5% of the total number of myonuclei.

Effects of Denervation on Myonuclear Number. Denervation resulted in a loss of myonuclei in old but not in young-adult muscles. This loss of myonuclei might be due to apoptosis, which has been shown to be more elevated in old than young-adult denervated muscles.[26] At the same time, however, the number of satellite cells increased more rapidly in old than in young-adult muscles. Although the role of the elevated number of satellite cells during short-term denervation is unknown, it has regularly been observed in both young-adult[45,46] and old denervated muscle.[20] It is possible that, although some myonuclei undergo apoptosis, other myonuclei are incorporated in the muscle fibers, thus resulting in an increased myonuclear turnover rate. If so, during denervation, the satellite cells must not only proliferate but also differentiate to donate their nuclei to the muscle fiber. Accordingly, it has been found that myogenin, a myogenic regulatory factor involved in satellite cell differentiation (see Zammit et al.[47] for review), is elevated in rat gastrocnemius muscle denervated for less than 4 weeks.[26,27,48] In addition, in 2–4-week denervated rat soleus muscles, an increased fraction of 5′-2′-bromodeoxyrudine-positive myonuclei was found.[27] Herein we observed an increased proportion of central myonuclei after 1 week of denervation. Although the function of central myonuclei is not completely clear, they are generally considered to be indicators of regeneration and to originate from differentiated satellite cells that have fused with the muscle fiber.[49] The faster increase in the number of satellite cells and the higher proportion of central myonuclei in old compared with young-adult gastrocnemius muscle suggests that the myonuclear turnover was higher in old than in young-adult denervated muscles.

Effects of Denervation on Myonuclear Domain Size. Although in old muscles denervation-induced atrophy was associated with a loss of myonuclei, the time-course of the decrease in myonuclear domain size did not differ between the young-adult and old muscles. For both ages a progressive decrease in domain size was apparent at 1 week of denervation and remained lower thereafter. This corroborates findings showing that short-term (7–21 days) and long-term (>2 months) denervation was associated with a decrease of myonuclear domain size in skeletal muscles from adult mice[7,8] and rats.[5] These observations indicate that muscle fiber size and the number of myonuclei are not necessarily coupled, as predicted by the concept of the constant myonuclear domain.[1] For several reasons it is unlikely that the decrease in myonuclear domain size during denervation is caused by apoptosis lagging behind the decrease in FCSA. One of these reasons is that nuclear apoptosis is a rapid process[50] that can be executed quickly, as reflected by the detection of apoptotic nuclei as early as 12 hours after the onset of hind-limb suspension in rat soleus muscle.[51] Nevertheless, even if apoptosis lagged behind atrophy, an increase in the myonuclear domain size would be expected between 2 and 4 weeks of denervation, the period in which no further atrophy occurred. This we did not observe. It is thus unlikely that the atrophy in this study was initiated by a disproportional reduction in the number of myonuclei. In fact, the decrease in RNA per myonucleus at 4 weeks of denervation indicates that the myonuclei in the denervated muscles did not use the full potential of their transcriptional capacity, which might be recruited when the muscle is subjected to a hypertrophic stimulus.

Effect of Denervation on Transcription and Translation. As there is no disproportionate loss of myonuclei, and hence no reduction in transcriptional capacity, other mechanisms must have induced an increase in protein degradation and/or decrease in protein synthesis to cause the denervation-induced atrophy.

It has been observed that the RNA content of denervated muscle is transiently elevated.[39] Herein we have extended these observations and found that in both young-adult and old denervated muscles the total RNA concentration was transiently elevated at 1 week of denervation, after which it returned to control levels. The elevated RNA concentration is most likely the result of a larger transcriptional activity per myonucleus. Because more than 80% of RNA is ribosomal RNA,[35] this supports earlier observations of an increase in the number of ribosomes in denervated muscle,[52] at least transiently. The increased ribosomal number is indicative of an enhanced translational capacity. This enhanced translational capacity might result in an increased rate of protein synthesis. A transient increase has in fact been observed at 1 week after the onset of denervation in extensor digitorum longus and soleus muscle of growing rats.[39] Because denervation has been shown to initially induce protein degradation and reduce or almost inactivate protein synthesis,[39] it is tempting to speculate that the transiently increased ribosomal capacity is utilized to increase the synthesis of enzymes involved in protein degradation. A significant increase in the activity and/or abundance of these enzymes is required to realize the 25% atrophy during the first week of denervation. Accordingly, it has been shown that the transcription of genes that regulate protein degradation, so-called atrogenes, and the expression of the ubiquitin protein are strongly elevated at 3–7 days after onset of denervation.[53–55]

In addition, we observed that the RNA content per myonucleus decreased in response to 2 and 4 weeks of denervation. If the half-life of RNA remained constant, this decrease would reflect a reduction of the transcriptional activity of the remaining myonuclei. If so, this may have contributed to the decrease in the rate of protein synthesis, which in turn would contribute to the denervation-induced atrophy.

In conclusion, in both 5- and 25-month-old rats, the majority of the denervation-induced atrophy of the gastrocnemius muscle occurred during the first 2 weeks. Although the old muscles lost some myonuclei, the myonuclear domain size decreased in both young-adult and old rats. It is therefore unlikely that the initial denervation-induced atrophy is due to a loss of myonuclei. In fact, there was an increase in the number of satellite cells and a transient rise in the total RNA content. The latter might reflect a temporary increase in ribosomal capacity. If a similar time-course of adaptations also applies to human conditions associated with denervation, such as spinal cord injury, it is advisable to start treatment of atrophy as soon as possible, when the number of ribosomes and satellite cells is still elevated and full-scale atrophy and loss of myonuclei have not yet taken place.

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